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Newport
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NP10 8QQ

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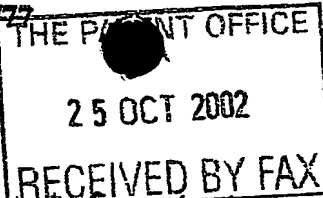
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The Patent Office

 Cardiff Road
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1. Your reference

P1643

2. Patent application number
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25 OCT 2002

0224872.2

3. Full name, address and postcode of the or of each applicant (underline all surnames)

 Sense Proteomic Limited
 The Babraham, Babraham Hall
 Babraham, Cambridge
 CB2 4AT

Patents ADP number (if you know it)

08238487002

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

4. Title of the invention

PROBE FOR MASS SPECTROMETRY

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

 Stratagem IPM Ltd
 The Old Rectory
 Nedging Tye
 Ipswich
 IP7 7HQ

Patents ADP number (if you know it)

07995780001

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Country

Priority application number
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Number or earlier application

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Description

27

Claim(s)

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Abstract

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Statement of inventorship and right to grant of a patent (Patents Form 7/77)

Request for preliminary examination and search (Patents Form 9/77)

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11.

I / We request the grant of a patent on the basis of this application.

Signature *Shragam Ipm Ltd* Date 26 Oct. 02

12. Name and daytime telephone number of person to contact in the United Kingdom

Dominic Schiller 0870 161 1673

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Probe for mass spectrometry

The present invention relates to a probe for the analysis of one or more analytes, particularly proteins or compounds capable of binding or otherwise interacting therewith, by laser desorption/ ionisation mass spectrometry, more particularly MALDI MS; It also relates to a protein microarray, a method of producing a protein microarray and a method of analysing a protein microarray.

Such a mass spectrometry probe, upon which a microarray has been fabricated, enables interrogation of protein - small molecule interactions in a label-free manner by desorption and ionisation of analytes (e.g. protein, drug or drug candidate, carbohydrate, DNA, RNA or other test molecule). The probe and methods are particularly useful in the drug discovery process, for example in hit series evaluation, lead optimisation, predictive toxicogenomics and metabolite profiling.

Analysis of disease processes and drug effects have traditionally focussed on genomics, whereas proteomics, the study of the expressed fraction of a genome, offers a more direct analysis of proteins and their inter-action. Proteomics was initially the quantitative and qualitative study of whole cell, tissue, organ or organism protein expression or fractions thereof. Often it involves comparing samples of similar biological origin exposed to different conditions or comparing diseased and non-diseased tissue. One advantage of proteomics over genomics is that it allows quantitative identification and analysis of proteins; by contrast, genomics can only predict the presence of proteins on the basis of mRNAs that might be translated into proteins. Furthermore, proteomics can identify posttranslational modification of proteins and can therefore draw conclusions about the activity of proteins rather than merely describing its presence.

Conventional analytical methods in proteomics are based on 2D-gel electrophoresis for protein separation followed by proteolytic digestion of the proteins and analysis by mass spectrometry. Alternatively Edman degradation can be used for protein identification after separation. However, both methods suffer limitations due to their bias towards highly expressed proteins and the destructive method of separation. Therefore proteomic methods which avoid the need for 2D-gel electrophoresis, such

as isotope coded affinity tag (ICAT, Gygi et al. 1999), tandem affinity protein purification (TAP, Gavin et al. 2002) and protein microarrays (McBeath and Schreiber, 2000), are gaining popularity. Furthermore, these new methods have broadened the scope of proteomics from collecting and cataloguing data to a stage where relations between molecules can be assigned; this is now referred to as functional proteomics.

Protein microarrays have most commonly taken the form of collections of immobilised antibodies that can be used, for example, to monitor protein expression levels in a miniaturised ELISA format (Schweitzer et al. 2002). The use of protein microarrays to analyse the function, rather than simply the abundance, of the immobilised proteins have received limited attention but recent examples include the analysis of substrate specificity within a set of yeast kinases (Zhu et al. 2000) and the identification of calmodulin- and phospholipid-binding proteins within a proteome-scale collection of yeast proteins (Zhu et al. 2001).

To date, protein microarrays have been analysed by enhanced chemo-luminescence (ECL), fluorescent or radioactive labels or via antibody based detection systems, but not by mass spectrometry. The current methods of analysing protein microarrays are therefore restricted by the availability of appropriate labelled ligands. Examples of labelled ligands that have been used successfully include fluorescently-labelled antibodies and radio- or fluorescently-labelled small molecule ligands. However, for drug-like small molecules, which often have molecular weights of less than 1000 Da, neither radio- or fluorescent labels are desirable; radiolabels are disfavoured for health and safety reasons, whilst the introduction of a fluorophore into the small molecule could significantly perturb the structure activity profile in an unpredictable manner. It is therefore clear that a label-free method to detect interactions in a microarray format would be a major advance and would greatly broaden the range of applications to areas where labelled compounds are not available or where labelling would alter the properties of the ligand. This would be particularly useful in the early stage of drug discovery, where great numbers of compounds are screened against proteins.

Amongst the label-free detection methods that are currently available, mass spectrometry has the unique advantage of being able to determine not only the

presence but also the identity of a given ligand. However, the development of a MALDI MS-compatible protein microarray is complex since existing methods for forming protein microarrays do not transfer readily onto to a MALDI target. There are a number of reasons why this is the case, inter alia the specialised nature of the probe surfaces and the potential for salts present in reaction buffers to interfere with the detection method. In addition, procedures known in the art for MALDI typically require the co-crystallisation of the aqueous analyte with acidic energy absorbing molecules, or 'matrix', to promote ionisation of the analytes (Karas and Hillenkamp, 1988). The method of co-crystallising analyte and matrix for MALDI, as known in the art, typically results in a heterogeneous crystallisation process and yields discrete, spatially separated crystals that each contain differing amounts of matrix and analyte. As a consequence it is often observed that individual crystals contain insufficient analyte for analysis by MALDI. This in turn results in a requirement for the analyser to sample multiple (ie. 10-100 or more) discrete locations within a given target area in order to obtain a good analyte signal; this is sometime referred to as "the search for the sweet spot" and imposes a significant lower limit on the size of individual target areas that can be routinely interrogated by MALDI MS methods known in the art.

In order to generate MALDI MS-compatible protein microarrays, solutions for the aforementioned shortcomings of the prior art are required that enable both miniaturisation of the target areas and functional analysis of the arrayed proteins.

Some examples of the affinity capture of analytes for mass spectrometric analysis have been described to date. However these examples relate to the use of single antibodies, nitriloacetic acid, anion exchangers or cation exchangers immobilised on the surface of the MALDI target or the use of bead based affinity capture reagents (Hutchens and Yip, 1993, Brockman and Orlando 1995, Wang et al 2001). However, all these methods suffer from one or more of the following limitations:

- a) Partial or total loss of biological activity because of amine-based coupling of the analyte or the bait onto the probe;

- b) Low specificity between the analyte and the surface which can lead to the non-specific binding of several analytes to the surface (e.g. ion-exchange surfaces);
- c) Low affinity of the analyte to the surface which can lead to leaching of the analyte from the surface during any wash procedures (e.g. ion-exchange and nitriloacetic acid surfaces);
- d) The affinity capture surface lacks non specific protein resistance, which can lead to high levels of non-specific protein binding which would interfere with the analysis of a protein microarray;
- e) The availability of only a limited number of affinity capture proteins.

Thus existing methods do not enable the immobilisation of large numbers of different, purified proteins in the form of a MALDI MS-compatible microarray suitable for functional analysis of the microarrayed proteins.

Summary of the Invention

The primary object of this invention is the development of a probe for the production of a protein microarray (as opposed to an array) which can be interrogated by means of laser desorption/ ionisation mass spectrometry, particularly matrix assisted laser desorption/ ionisation (MALDI).

The invention also relates to methods leading to the production of such a probe, a protein microarray which can be interrogated by means of laser desorption/ ionisation mass spectrometry, particularly matrix assisted laser desorption/ionisation (MALDI) and methods of analysing such a probe or protein microarray.

Some of the significant advances leading to the development of such a probe are described in Applicant's co pending application WO 01/57198 and are thus not dealt with in depth herein.

However, the development of a MALDI MS-compatible protein microarray is complex since existing methods for forming protein microarrays do not transfer

readily onto to a MALDI target. There are a number of reasons why this is the case, including the specialised nature of the probe surfaces and the potential for salts present in reaction buffers to interfere with the detection method.

Procedures known in the art for MALDI typically require the co-crystallisation of the aqueous analyte with acidic energy absorbing molecules, or 'matrix', to promote ionisation of the analytes (Karas and Hillenkamp, 1988). The method of co-crystallising analyte and matrix for MALDI, as known in the art, typically results in a heterogeneous crystallisation process and yields discrete, spatially separated crystals that each contain differing amounts of matrix and analyte. As a consequence it is often observed that individual crystals contain insufficient analyte for analysis by MALDI. This in turn results in a requirement for the analyser to sample multiple (ie. 10-100 or more) discrete locations within a given target area in order to obtain a good analyte signal; this is sometime referred to as "the search for the sweet spot". This has prevented miniaturisation since protein spots need to be large. They are generally in the order of at least 0.5mm^2 .

In order to generate MALDI MS-compatible protein microarrays, solutions for the aforementioned shortcomings of the prior art are required that enable both miniaturisation of the target areas and functional analysis of the arrayed proteins.

As defined herein a probe is a support which is capable of acting as a target in analysis by laser desorption/ionisation mass spectrometry, for example matrix assisted laser desorption/ionisation (MALDI). The probe carries the analytes, for example proteins, during such processes and interacts with the repeller lens of the ion-optic assembly found in laser desorption/ionisation time-of-flight (TOF) mass spectrometers of the art, such that the analytes are converted to gaseous ions to permit analysis. For example, the probes of the invention may be derived from targets for MALDI analysis as known in the art, which are treated such that a high affinity protein binding moiety e.g. streptavidin, avidin or neutravidin molecules are present on the probe surface which bind biotinylated proteins for subsequent analysis. For example, conventional glass or gold MALDI targets may be used.

As defined herein a micro array is an array where the size of the discrete target areas i.e the individual areas probed by a laser, is in the order of micrometers or less .

Whilst at the upper end of the scale, around 1000 micrometers diameter, they may be visible to the naked eye at the lower end of the scale the discrete target areas will not be clearly distinguished by the naked eye.

The arrays will typically be arranged in matrices comprising several rows and columns. The number of discrete target areas will depend upon what is being screened though it is generally desirable to have a high density of these discrete areas on the probe surface as this will facilitate high throughput screening. Typically a probe will comprise at least 10, more preferably at least 100, more preferably at least 1000 and as many as 10,000 or more target areas produced thereon. (Typically a probe surface will have an area of around $10,000\text{mm}^2$ - a Bruker probe has an area of 10292mm^2 although there is no requirement to use the whole of the probe and the microarray can be applied in one or more matrices thereon.) The actual density in a given matrices will depend upon the size of the discrete target area (which will typically be printed as a spot) and the spacing between adjacent spots. Thus the discrete target areas will typically be present at a density of greater than 1 discrete target areas per mm^2 within any matrices.

An analyte capture moiety is the moiety which captures the component which is being screened. Preferably, though not essentially the capturing element is a protein although it is possible to have an array in which, for example, small molecules are bound to the surface and thus to screen for proteins.

The term proteins as used herein is used to include both whole proteins and sub units or domains thereof.

Fusion protein as used herein is used to refer to a protein, which has a tag, for example, a biotinylation consensus sequence or phleomycin/zeocin resistance binding protein attached thereto.

Linker molecules are molecules which function as their name suggests. They are molecules comprising functional groups which allow bridges to be formed between different molecules.

According to a first aspect of the invention there is provided a probe, for use with a laser desorption/ ionisation mass spectrometer, comprising a support having an electroconductive target surface thereon characterized in that the target surface comprises a micro array having a plurality of discrete target areas presenting one or more analyte capture moieties.

The development of such a probe will enable high through put screens to be conducted and a plurality of protein interactions to be studied.

Another significant development enabling the "miniaturisation" of a protein array formed on a MALDI target derives from the application of the Applicant's COVET technology described in WO 01/57198. Briefly, using this technology they are able to create from cDNA libraries expressed proteins, which carry a "sequence tag" that can be used to capture the proteins with a high affinity and in a specific orientation on the microarray surface. This firstly enables proteins e.g. a protein library to be stably immobilized such that leaching of protein from the surface is avoided and secondly the oriented immobilisation of the fusion protein onto the surface ensure maximum biological activity.

Yet another significant aspect of the invention, when compared to current protein microarrays, is the provision of such a probe with an electro conductive surface. This surface which includes semi conductive surfaces is essential where the probe is to be subjected to MALDI MS analysis. Whilst the support could be made wholly of an electro conductive material (which term is used herein to include semi conductive materials) it is preferred to coat a rigid support, e.g. a glass, with an electro conductive material such as, for example, gold although any suitable metal, for example, silver, platinum, Iridium, wolfram, copper, cobalt, nickel, and iron or mixtures thereof, or a semiconductor e.g. silicon oxide, graphite or germanium oxide could be used.

Where the probe or protein microarray is produced on e.g. a standard size microscope glass slide it can be mounted in an adapter, which carries it into a mass spectrometer. Such an adaptor is described in Applicant's co pending UK application no. 0216387.1.

A further significant development, and one which may be viewed independently of the specific applications described herein, has been in the way the Applicant has overcome the problems caused by non specific protein binding. The Applicant has overcome this particular problem by providing a layer resistant to non specific protein binding onto the probe surface. More particularly, the microarray surface is modified by the inclusion of a layer of molecules which repel proteins. These protein repellant molecules which include, for example, polyethyleneglycol may be bound to the probe surface via a linker, such as, for example, a poly amino acid which readily binds to e.g. a glass or gold surface and whose amino or carboxyl side groups can be used to bind the protein repellant molecules such that they reach out from the probe surface. The skilled man will appreciate that other functionalized molecules could be used. Preferably the analyte binding moieties are incorporated in a position where they extend out from the surface. Preferred protein binding moieties include e.g. biotin, biotin-neutravidin, and bleomycin, and these and other moieties can be incorporated into the layer either via these functional groups on the linker molecules and/ or via functional groups on the protein repellant molecules. Typically the affinity capture moieties are incorporated in small proportions (typically less than 20%) relative to the protein repellant molecules.

In this way the Applicant has been able to introduce the protein capture moieties not only in a homogeneous, spatial defined arrangement but also in a manner which enables high affinity binding in a specific manner. The resulting surface combines selectivity for the capture of biological macromolecules on the probe with reduced non specific binding of the type commonly observed on underivatized glass or metal surfaces and additionally results in a homogeneous distribution and orientation of the captured biological macromolecules.

The component molecules responsible for repelling non specific proteins include molecules which are generally hydrophilic in nature. They include polymers, such as,

for example, polyethylene glycol, dextran, polyurethane and polyacrylamide and self assembled monolayers (SAM). Preferably the polymers comprise one or more functional side groups via which the protein capturing moieties can be attached. In the case of polyethylene glycol the functional group is a hydroxyl group. The molecules responsible for repelling non specific proteins may be bound directly to the surface as in, for example the case of SAM's or they may be attached via a linker. Particularly preferred as linkers are poly amino acids such as, for example, poly L lysine, poly L aspartic acid, poly L glutamic acid or mixtures thereof. These have amino or carboxy side chains via which the molecules responsible for repelling non specific proteins can be attached and which can additionally be used to attach the protein capturing moieties. Alternatively, or in addition, the protein capturing moieties can be attached via the component molecules responsible for repelling non specific proteins. Fig 7 illustrates the binding of such molecules and contrasts the defined orientation which can be achieved by this ordered coupling compared to that achieved using current antibody binding techniques which result in random coupling.

In a preferred embodiment the probe has as it's protein capture moieties either a biotin binder e.g. neutravidin, avidin or streptavidin or a bleomycin resistant protein binder e.g. bleomycin. The proteins are bound to the probe to create a protein microarray by printing a plurality of bacterial, yeast, sf9 or mammalian cell lysates containing fusion proteins in which a high affinity tag e.g. biotin or zeocin resistant protein (ZRP) is expressed onto the capture surface. Proteins are derived from the expression of a cDNA library and each individual clone is tagged at the C-terminus and/ or on the N-terminus with a consensus sequence, which will enable high affinity recognition of the protein even in the presence of the otherwise protein repellent molecules. Only the recombinant, tagged protein can recognise the capture surface and other proteins from the lysate can be washed away as they do not bind to the protein repellent surface and do not have a high affinity to the protein binding moieties present in the layer.

Another aspect of the invention is the study of the full protein complement, or a significant fraction thereof, of given cell or tissue type using a probe or protein microarray according to the invention.

According to a further aspect of the present invention there is provided a method of producing a protein microarray for use with laser desorption ionisation mass spectrometer comprising providing a probe of the invention and depositing protein in registration with the protein capturing moieties in the discrete target area.

According to a further aspect the invention utilizes the probes and protein microarrays to analyse and screen various reactions.

One method of analysis by laser desorption/ionisation mass spectrometry comprises the steps of:

- a) providing a probe of the invention;
- b) bringing said probe into contact with one or more proteins; and
- c) performing laser desorption/ ionisation mass spectrometry on the proteins on the surface of the probe.

In one embodiment the method comprises, between step b) and c), an additional step of removing unbound molecules from the probe by washing.

In another embodiment the one or more proteins are contained in a mixture of proteins.

In yet a further embodiment, which is a method for identifying a protein on the surface of the probe, the method comprises the additional steps of:

- d) determining the mass of the protein molecule;
- e) performing a digestion upon a replicate sample of said protein on a further probe or probe surface; and
- f) performing laser desorption/ ionisation mass spectrometry on the peptides resulting from step e) to identify said protein(s).

In another embodiment there is a method for analysing the function of a protein on the surface of the probe and a molecule interacting with said protein and which comprises the alternative and additional steps of:

- c) bringing a protein on the probe surface into contact with one or more test molecules;

- d) removing unbound test molecules from the probe surface;
- e) performing laser desorption/ ionisation mass spectrometry on the protein and any molecule that had been specifically retained on the probe surface through interaction with the protein to determine the identity of the protein and/or test molecule.

The test molecule may be a small molecule, protein, or a nucleic acid e.g. DNA or RNA.

In a further embodiment there is a method for analysing the function of a protein on the surface of the probe and a molecule interacting with said protein and which comprises the alternative and/or additional steps of:

- c) bringing a protein on the probe surface into contact with one or more test substrates; and
- d) performing laser desorption/ ionisation mass spectrometry on the protein and test substrates to determine the presence and/or identity of products of catalysis of said test substrates by the protein.

In one embodiment a cDNA library which has been cloned to express a high affinity tag is expressed and after expression of each clone, the tagged library proteins are captured by the protein affinity moieties and dried onto the microarray, overlaid with a proteolytic agent of biological or chemical origin, cleaved into fragments, overlaid with energy absorbing matrix molecules prepared in a non-aqueous solvent that is spiked with and anti evaporative agents such as glycol. The energy absorbing molecules are applied to the protein microarray in a new formulation at volumes of e.g. a few nanoliters to form a continuous layer of microcrystals.

This use of energy absorbing molecules in this way is yet another and independent aspect of the invention.

According to a further aspect of the present invention there is provided a method of analysing a probe of the invention in which energy absorbing molecules are deposited in a manner which denatures and thus unbinds a protein from a protein capturing moieties leaving the denatured protein lying unbound on the surface.

The energy absorbing molecules form a homogenous layer of crystals in a discrete locations in registration with the protein capturing moieties and captured protein.

The homogenous layer of crystals is substantially continuous such that individual crystals are not visible at a 100 fold magnification and there are no visible gaps. It also has a substantially uniform depth, such that there is no apparent variation in crystal size at a 100 fold magnification.

The energy absorbing molecules are deposited onto the surface in a non aqueous solvent and the non aqueous solvent is evaporated off. Preferably the non aqueous solvent is an organic solvent, such as, for example, acetone or butanone.

Preferably the non aqueous solvent includes a modifier which controls the rate of evaporation such that crystallisation of the energy absorbing molecules occurs on the probe. Suitable modifiers include glycerol, polyethyleneglycol and thioglycerol. Preferably the energy absorbing molecules are deposited in a mixture of from 80 - 99.9%, preferably 99% organic solvent e.g. acetone to 20 - 0.1%, preferably 1% of modifier e.g. glycerol (vol/vol). Typical energy absorbing molecules include crystals of α -cyano-4-hydroxy-cinnamic acid, sinapibic acid, gentisic acid, nifedine, succinic acid, 1,8,9,-anthracenitriol, 3-Indoleacrylic acid, 2-(hydroxyphenylazo) benzoic acid, 4-nitroanilin and combinations thereof.

Preferably the energy absorbing molecules are deposited in registration with the protein and each protein spot is overlaid with a similar sized spot of the energy absorbing molecules.

A further application of the protein microarray is the parallel analysis of protein-protein, protein-nucleotide and protein small molecule interaction by mass spectrometry.

Yet another aspect of the invention is its usefulness to screen small molecule compound libraries on the probe to detect binding of drug-like small molecules to proteins that are derived from a proteome, where the small molecules do not carry a label such as a radiolabel or a fluorescent label.

In order to achieve a high density of individual samples on the microarray the energy absorbing molecules need to be arranged in microcrystals on the surface. The matrix forms a homogenous layer of flat crystals without significant gaps between them and can be deposited in very small quantities on the microarray.

In contrast to the prior art in which matrix and analyte are co-crystallised in an aqueous solvent, the Applicant uses two distinct steps in which first the protein is deposited in an aqueous solvent and then the energy absorbing molecules are deposited such that they crystallise out from the non aqueous solvent on the probe. This has the advantage that the protein is deposited in its biological form. However, using a non aqueous solvent to deliver the energy absorbing molecules allows the formation of a homogenous layer of microcrystals. This has two benefits. First the formation of a homogenous layer means it is not necessary to search for a sweet spot as the homogenous layer guarantees protein in the presence of energy absorbing molecules and secondly it results in more accurate measurement due to the even nature of the layer.

Another aspect of the invention is the automated analysis of small molecules binding to proteins present on the microarray. The molecular weight of small molecule ions, which are stored in a database can be compared with the measured molecular weight of a compound library and therefore the relationship between the small molecule and protein in the array can be assigned.

The various aspects of the invention will now be described, by way of example only, with reference to the following figures and examples in which:

Fig 1a show six screenshots taken from a Bruker Autoflex mass spectrometer flexcontrol tool comparing the crystal surface of one aspect of the invention with that obtained practicing the method of the prior art. The six screenshots show three different matrices prepared in two different ways.

On the left side (top to bottom) are:

- i) α -cyano-4-hydroxy-cinnamic acid;
- ii) sinapinic acid; and
- iii) gentisic acid.

All have been prepared in 99% acetone, 1% glycerol (v/v).

On the right hand side (top to bottom) are the same matrices

- iv) α -cyano-4-hydroxy-cinnamic acid;
- v) sinapinic acid; and
- vi) gentisic acid.

prepared in aqueous solvents as per the prior art.

Fig 1b shows a photomicrograph of α -cyano-4-hydroxy-cinnamic acid crystals. The matrix was dissolved in 99% acetone w/v, 1% glycerol and arrayed onto a gold coated glass slide with an affinity capture surface. The printing density is 562 micrometers from spot center to spot center.

Fig 2a shows a mass spectrum acquired from a protein microarray demonstrating the capture of 1500 femtogram insulin-biotin on a affinity capture surface. There are three insulin-biotin peaks visible due to different degree of biotinylation. Up to 3 biotin molecules were observed on insulin in the range of 6000 dalton. Two additional peaks are observed at 7300 dalton and 14600 dalton and are assigned as Neutravidin $[MH]^+$ and $[MH]^{2+}$.

Fig 2b shows a mass spectrum acquired from a protein microarray demonstrating the capture of 15 femtogram insulin-biotin on a affinity capture surface. Two insula-

biotin peaks are visible in the area of 6000 dalton. Two additional peaks are observed at 7300 dalton and 14600 dalton and assigned as Neutravidin $[MH]^+$ and $[MH]^{2+}$.

Fig 3a shows the detection of Cyclosporin by mass spectrometry on a PEG-PLL-Biotin Neutravidin affinity capture surface. Cyclosporin is detected at 1205 dalton and Neutravidin $[MH]^+$ and $[MH]^{2+}$ peaks are present at 7310 and 14652 dalton.

Fig 3b shows the detection of Ketoconazole by mass spectrometry on a PEG-PLL-Biotin Neutravidin surface. Ketoconazole is detected at 534 dalton and Neutravidin $[MH]^+$ and $[MH]^{2+}$ peaks are at present at 7325 and 14501 dalton.

Fig 3c shows the detection of Quinidine by mass spectrometry on a PEG-PLL-Biotin Neutravidin surface. Quinidine is detected at 327 dalton and Neutravidin $[MH]^+$ and $[MH]^{2+}$ is present at 7310 and 14652 dalton.

Fig 4a shows the detection of ADP and ATP. ATP was enzymatically synthesized from the reaction of ADP, creatine phosphate and creatine phosphate kinase in 25 mM ammonium bicarbonate at pH 7.4. $[ADP]^+$ was detected at 427.6 dalton and $[ADP+Na]^+$ 449.6 dalton. The products of the creatine phosphate kinase reaction were detected at 507.6, 529.6, 551.6 and 573.8, which fits well with the expected molecular weight of $[ATP]^+$ and three ATP sodium adducts $[ATP Na]^+$, $[ATP Na_2]^+$ and $[ATP Na_3]^+$. Control reactions in which either one of the substrates ADP or creatine phosphate or the enzyme creatine phosphate kinase was omitted didn't show ATP peaks.

Fig 4b shows a MALDI mass spectrum detecting human cytochrome p450 oxidation products of dibenzylfluoresceine (DBF). DBF was oxidized by cytochrome P450 and a metastable oxidation product was detected at 530 dalton. Further molecular ions of oxidized dibenzylfluoresceine were detected at 477 and 461 dalton presenting two monobenzyfluoresceine derivatives.

Fig 5 shows the capture of a biotinylated 72 Kda polypeptide on a PEG-PLL-Biotin Neutravidin coated gold target. The protein was expressed in 200 microliter *Escherichia coli* culture, the bacteria were lysed with lysozyme and Dnase treated. The resulting bacterial lysate was spotted onto a affinity capture surface and incubated

for 4 hours. The probe was then washed with 1 mM Tris-HCl pH 7.5 0.1% Triton followed by two washes with 1 mM Tris-HCl pH 7.5 for desalting and removal of detergent. The probe target was then dried under nitrogen and overlaid with energy absorbing matrix (α -cyano-4-hydroxy-pinnonic acid dissolved in acetone). The mass spectrum was acquired in linear mode using the delayed extraction technique at low laser power.

Fig 6 shows identification of genetically engineered *Schistosoma mansoni* Glutathione-S-Transferase BCCP fusion protein that was expressed in *Escherichia coli*. Glutathione-S-Transferase was captured from a crude bacterial lysate on the probe by the use of affinity capture polymers. The captured analyte was washed and digested on the probe overlaid with energy absorbing matrix dissolved in acetone and analysed by a MALDI TOF mass spectrometer. The resulting peptide masses were used for a protein fingerprint analysis and the fusion protein was identified as Glutathione-S-Transferase from *Schistosoma japonicum*.

Fig 7 shows random and orientated coupling of proteins on a probe for example a MALDI target, microtiter plate or a microscope glass slide.

Figure 8. The binding of poly-L-lysine poly ethyleneglycol biotin polymer (PEG-PLL-biotin) to a biosensor is shown. Subsequently, neutravidin and a protein lysate from *E. coli* containing biotin tagged Glutathione-S-transferase (GST-BCCP) was added to the surface followed by a washing period for each step.

Figure 9a and 9b Mass spectra of distinct forms of the glycoprotein Fetuin on immobilised lectins. (a) Biotinylated peanut lectin was immobilised on a PEG-PLL-biotin-neutravidin surface for the capture of the glycoprotein Fetuin. The $[M+H]^+$, $[2M+H]^+$ molecular ions of the lectin were observed at 25774 and 51461 dalton and the $[M+H]^+$ molecular ion of neutravidin was observed at 14300 dalton. Peaks accounting for the molecular ions of the glycoprotein were observed at 40136 and 42731 dalton. (b) Biotinylated wheat germ agglutinin was immobilised on PEG-PLL-biotin-neutravidin surface and used for the specific capture of the glycoprotein Fetuin.

The $[M+H]^+$, $[2M+H]^+$ molecular ions of the lectin were observed at 17709 and 35584 daltons and $[M+H]^+$, $[2M+H]^+$ molecular ions of neutravidin were observed at 14300 and 28600 dalton. The $[M+H]^+$ molecular ion of the glycoprotein Fetuin was observed at 44163 dalton and two peaks at 25943 and 32158 daltons were specific for the glycoprotein and represent most likely breakdown products.

Fig 10a Figure 5 shows the binding the specific binding of a Rhodamine-lactose derivative to the lectin from *Arachis hypogea*. (a) A PEG-PLL-Neutravidin *Arachis hypogea* surface was overlaid with 1 mM lactose-rhodamine conjugate and washed three times with 1 mM Tris-HCl, pH 7.5 and overlaid with a solution of energy absorbing α -cyanohydroxycinnamic acid dissolved in acetone. The following MALDI MS analysis shows a molecular ion at 830.32 dalton which fits with $[MH]^+$ of lactose-rhodamine.

Fig 10b Shows the MALDI MS analysis of the lactose-rhodamine conjugate as used in the experiment. The lactose-rhodamine molecular ion is detected as well as the sodium adduct molecular ion at 834 dalton.

Fig 10c A PEG-PLL neutravidin surface with the immobilised FK506 binding protein was overlaid with a 1 mM lactose-rhodamine conjugate and washed three times with 1 mM Tris-HCl pH 7.5 and overlaid with energy absorbing matrix molecules dissolved in acetone. The MALDI MS analysis shows no molecular ions of lactose rhodamine.

Table 1 shows the molecular weights of peptides which could be assigned to three protein by protein fingerprint analysis of a Glutathione-S-transferase digest. The molecular weights of the peptides were used to search NCBI nr database using the MASCOT search engine with a mass accuracy of 50 ppm. The matched proteins are glutathione-S-transferase, avidin and trypsin.

Detailed description

1. Preparation of a probe according to one aspect of the invention.

1.1 Cleaning of gold coated glass slide and MALDI probe.

A probe comprising a gold coated microscope glass slide or a MALDI probe was thoroughly cleaned before use with sequential washing steps in acetone, acetonitrile, double distilled water and dried under nitrogen.

1.2 Non protein binding layer incorporating protein binding moieties prepared and deposited

1.2.1 PEG-PLL derivative synthesis

PEG-PLL-Biotin:

100 mg poly-L-lysine average size 17-30 kda (Sigma, Dorset, UK) was reacted with 109 mg mPEG-SPA (Shearwater Corporation, Huntsville, Alabama) and 1.1 mg biotin PEG-CO-NHS in 100 mM carbonate buffer pH 9 for a period of 30 minutes. The reaction was terminated by dialysis in 1 mM Tris-HCl pH 7.5 over night. The product from this reaction was called 1% PEG-PLL-Biotin (1% PEG derivatives contain a biotin headgroup) and several other small ligand ratios were synthesized (1%, 2%, 10% and 20%).

PEG-PLL-Bleomycin:

10 mg of bleomycin B6 (Calbiochem) was dissolved in 1 ml acetone and 7.5 mg EDC and NHS each was added. The pH of the reaction was adjusted with HCl at pH 3. In another reaction 99 mg poly-L-lysine was reacted with 11 mg DVS-PEG-CO-NHS and 100 mg mPEG-CO-NHS in 100 mM carbonate buffer pH 9. After 20 min both reactions were mixed and the pH was adjusted to pH 9 when necessary. The PEG-PLL-Bleomycin synthesis was cleaned up by a dialysis against a plentiful amount of 1 mM Tris-HCl pH 7.5 buffer over night. The product of this synthesis was called 10% PEG-PLL-Bleomycin indicating that approximately 10% of the PEG side chains are substituted with Bleomycin.

Freshly prepared affinity capture polymer, for example, 1% PEG-PLL-Biotin or 1% PEG-PLL-Bleomycin B6 was deposited onto the probe. The surface was then covered with Nesco film to evenly distribute the protein capture moiety over the probe. After 30 min the probe was washed in 1 mM Tris-HCL pH 7.5 and dried under nitrogen.

The PEG-PLL-Bleomycin B6 surface was ready for use.

1.3 Alternative protein capture moiety added if required

The PLL-PEG-biotin has a neutravidin molecule bound to the biotin by adding 0.5 mg/ml neutravidin for one hour at RT in a humid chamber. The Probe was then rinsed with washing buffer, and washed twice with ample desalting buffer before it was dried under nitrogen. The surface was now ready to be used as a highly specific affinity capture of macromolecules carrying an appropriate affinity tag, e.g. Biotin or phleomycin/zeocin resistance binding protein.

2. Preparation of a protein microarray according to one aspect of the invention.

2.1 Tagged proteins produced

Purified mRNA from heart, liver or breast tissues are transcribed into cDNA using known techniques. The 3' end of the cDNA is made accessible to a 3' to 5' single-stranded exonuclease which digests one strand of the DNA. The reaction is controlled through manipulation of parameters such as time, temperature and salt concentration. The remaining single stranded region of DNA is then removed by a single-stranded nuclease such as mung bean nuclease, to leave a blunt end. The resulting truncated double stranded cDNA is then digested with a rare-cutting restriction enzyme which has a site at the 5' end of the cDNA, introduced during cDNA synthesis. The resulting cDNA fragment is then ligated to a DNA tag which encodes a marker of solubility. In this case, this is achieved by ligating the cDNA fragment into a vector which provides a tag 3' to the cDNA fragment. Transcription initiates upstream of the cloned cDNA and proceeds through the cDNA and downstream tag. When ligated in-frame and in the absence of stop codons, the resulting translation product consists

- i) α -cyano-4-hydroxycinnamic acid (Sigma, Dorset, UK) was dissolved in acetone at saturating amounts and 300 nanoliter of the solution was used to overlay the analyte.
- ii) Sinapinic acid (Sigma, Dorset, UK) was dissolved in acetone at saturating amounts and 300 nanoliter of the solution was used to overlay the analyte.
- iii) Gentisic acid (Sigma, Dorset, UK) was dissolved in acetone at saturating amounts and 300 nanoliter of the solution was used to overlay the analyte.
- iv) 10 mg/ml α -cyano-4-hydroxycinnamic acid (Sigma, Dorset, UK) was dissolved in 50 % v/v acetonitrile, 0.1% trifluoroacetic acid as known in the art and 300 nanoliter of the solution is used to overlay the analyte on the probe.
- v) Sinapinic acid (Sigma, Dorset, UK) was dissolved in ddH₂O at saturating amounts and 300 nanoliter of the solution is used to overlay the analyte.
- vi) Gentisic acid (Sigma, Dorset, UK) was dissolved in ddH₂O at saturating amounts and 300 nanoliter of the solution was used to overlay the analyte.
- vii) α -cyano-4-hydroxy-cinnamic acid (Sigma, Dorset, UK) is dissolved in 99% acetone v/v, 1% glycerol in saturating amounts. 3 nanoliter of the matrix formulation is then transferred onto the probe, which contains the analyte.

3.2 Generic method for microcrystallisation of energy absorbing matrix molecules

The three examples of energy absorbing molecules prepared as described in 3.1 above were arrayed onto a protein microarray and the appearance at 100 fold magnification is illustrated in Fig 1a. The acetone dissolved matrix i), ii) and iii) show a very homogenous crystal formation compared with the aqueous matrix iv), v) and vi) formulation currently used in the art.

Referring to Fig 1a the left hand side slides show the acetone dissolved formulations whereas on the right hand side the aqueous matrix formulation are shown.

The new matrix formulation illustrated have proved significant in being able to generate protein microarrays (see Figure 1b) because they allow a more efficient use of space on the probe surface, have enhanced flatness allowing greater mass accuracy, and furthermore increased amounts of matrix can be deposited on the probe to meet the needs of high analyte density.

Fig 1b illustrates a probe according to one aspect of the invention with protein captured thereon (thus forming a protein microarray) with an energy absorbing matrix according to a further aspect of the invention overlaid. The α -cyano-4-hydroxycinnamic acid matrix was dissolved in acetone 99% v/v, 1% glycerol v/v and arrayed onto a gold coated microscope slide. After solvent evaporation, matrix crystals are formed. In contrast to the crystals formed by the deposit of aqueous solutions the non-aqueous solvent formulation of matrix lead to a very homogeneous and flat crystal layer. Because of this the analyst looking at the spots can "hit" the analyte within the "spot" and consequently the spot can be made smaller enabling the miniaturization and production of a microarray because of the resulting high spatial density, which could not be created using aqueous matrix formulations. This is a significant development in the creation of mass spectrometric compatible protein microarrays.

4. Protein array subjected to MALDI and different methods of use.

4.1 Surface capture of substantially pure tagged biological macromolecules

Example 1

Affinity capture of a variety of tagged proteins can be demonstrated using for example PEG-PLL-biotin or PEG-PLL-Biotinmycin B6 as the protein capturing moieties.

Figs 2, a and b show the mass spectra acquired from a protein microarray demonstrating respectively the capture of 1500 and 15 femtogram of biotin tagged insulin. The biotin tagged insulin was arrayed onto an affinity capture surface on a gold coated microscope glass slide in a 3 nanoliter volume using 300 micrometer pins

4.3 Capture, detection and identification of recombinant protein on probe using a degradation process

Example 3

Figure 6 shows the peptide fingerprint analysis of Glutathione-S-transferase -Biotin Carboxyl Carrier Protein (GST-BCCP). A bacterial crude lysate containing the fusion protein and bacterial proteins was placed on the MALDI target previously coated with PEG-PLL-biotin and neutravidin. The BCCP fusion partner of GST contained a biotinylation consensus sequence such that it becomes biotinylated when expressed in *E. coli*, allowing the fusion protein to bind specifically to the PEG-PLL-biotin neutravidin surface, whilst allowing the bacterial proteins to be washed away with buffer. For identification purpose the surface captured protein was digested by overlaying it with trypsin and analysed by MALDI MS. A protein fingerprint analysis revealed 12 peptides belonged to GST from *Schistosoma mansoni*, 4 peptides belonging to Neutravidin and 3 to trypsin (see table 1), but no bacterial protein was identified using the remaining un-matched peptides. This experiment demonstrates that PEG-PLL-biotin and neutravidin can be used to purify a protein from a crude mixture of protein in a single step on a MALDI target. Taken together this experiment paves the way for protein microarray production where the protein content on the array is derived from a bacterial expression system without the need for an initial pre-purification step.

Example 4

Figure 9a shows a mass spectrum of the biotinylated lectin from *Triticum vulgaris* (WGA) captured onto a PEG-PLL-Biotin Neutravidin surface. The lectin was then

4.4 Detection of small molecules on protein microarrays

To demonstrate the capability of small molecule detection in the presence of the PEG-PLL-biotin and Neutravidin three small molecules used in pharmacology and toxicology were spiked onto the array. The molecules Cyclosporin, Ketoconazole and Quinidine were identified at their corresponding molecular weight.

A PEG-PLL-biotin coated probe was incubated with a solution of Neutravidin and washed extensively with washing buffer (1 mM Tris-HCl pH 7.5 with 0.1% Triton X-100) and desalting buffer (1 mM Tris-HCl pH 7.5.), dried and overlaid with energy absorbing matrix and then analysed with MALDI TOF mass spectrometry.

The mass spectra (Figure 3a, 3b and 3c) show the specific capture of Neutravidin $[MH]^+$ and $[MH]^{2+}$ at 7310 and 14652 dalton.

Example 6

In a further example the binding of a small molecule to a protein is demonstrated in Figures 10a,b, and c. The lactose rhodamine conjugate was specifically retained on a PEG-PLL-Neutravidin Arachis hypogaea lectin surface whereas it could not be detected on a PEG-PLL-Neutravidin FK506 binding protein surface. This is another example for the detection of a small molecule protein interaction. The example is surprising since binding constant for lactose and this lectin is in the millimolar range, suggesting that the presence of the rhodamine moiety has increased the affinity of the small molecule ligand.

4.5 Detection of a reactant on a protein microarray

Example 7

ATP was enzymatically synthesized from the reaction of ADP, creatine phosphate and creatine phosphate kinase in 25 mM ammonium bicarbonate at pH 7.4. $[ADP]^+$ was detected at 427.6 dalton and $[ADP+Na]^+$ 449.6 dalton (see Fig 4). The products of the creatine phosphate kinase reaction were detected at 507.6, 529.6, 551.6 and 573.8, which fits well with the expected molecular weight of $[ATP]^+$ and three ATP sodium adducts $[ATP Na]^+$, $[ATP Na_2]^+$ and $[ATP Na_3]^+$.

Control reactions in which either one of the substrates ADP or creatine phosphate or the enzyme creatine phosphate kinase were omitted didn't show ATP peaks.

4.6 Detection of a reactant on a protein microarray

Example 8

The oxidation of drug-like small molecules by human cytochrome P450 enzymes is the usual first step in the metabolism of such compounds.

Here, the oxidation of dibenzylfluorescein by cytochrome P450 3A4 was studied with MALDI MS and the results illustrated in Fig 4. Dibenzylfluorescein (DBF) was detected at 513.795 [MH]⁺ and a metastable oxidation product was observed at 530.069, which indicates the addition of one oxygen. The resulting molecule is known to be chemically unstable and therefore monobenzylfluorescein (MBF) and their oxidation products can be observed at 444.912 [MH]⁺, 460.890 [MH+O]⁺ and 476.855 [MH+2O] dalton.

This experiments shows the suitability of a protein arrays to detect biological catalysis and to assign function to biological polypeptides captured on protein arrays.

The mass spectra from the figures listed below had been obtained on

1. Bruker Daltonic gold targets #26995 (Figure 3a, 3b, 3c, 4, 5)
2. Bruker Daltonic glass target #26754 (Figure 6)
3. Bruker Daltonic MTP 384 target milled out to harbor a gold coated microscope 30 x 75 mm glass slide (Figure 2a, 2b)

CLAIMS

1. A probe, for use with a laser desorption/ionisation mass spectrometer, comprising a support having an electroconductive target surface thereon characterized in that the target surface comprises a micro array having a plurality of discrete target areas presenting one or more analyte capture moieties.
2. A probe as claimed in claim 1 wherein the support is a glass slide, or a MALDI target.
3. A probe as claimed in claim 1 or 2 wherein the plurality of discrete target areas are arranged in a spatially defined manner.
4. A probe as claimed in any of the preceding claims wherein each discrete target area has an area of less than $1000\mu\text{m}^2$, more preferably still less than $500\mu\text{m}^2$, and more preferably still less than $100\mu\text{m}^2$.
5. A probe as claimed in any of the preceding claims wherein each discrete target area has an area of less than $785\mu\text{m}^2$, more preferably less than $392\mu\text{m}^2$, more preferably still less than $78\mu\text{m}^2$.
6. A probe as claimed in any of the preceding claims wherein the discrete target areas are substantially circular.
7. A probe as claimed in any of the claims wherein the discrete target areas are arranged in matrices.
8. A probe as claimed in claim 7 wherein there are a plurality of matrices on the target surface.

20. A probe as claimed in any of the preceding claims wherein the analyte capture moieties have a high affinity for their binding partner.
21. A probe as claimed in claim 20 wherein the binding affinity (K_d) between the analyte capture moiety and its binding partner is at least $10^{-7}M$, more preferably at least $10^{-9}M$, more preferably at least $10^{-12}M$, and more preferably still at least $10^{-15}M$.
22. A probe as claimed in any of the preceding claims wherein the analyte capture moiety is attached directly to the electroconductive target surface.
23. A probe as claimed in any of claims 1 to 21 wherein the analyte capture moiety is indirectly attached to the electroconductive target surface.
24. A probe as claimed in claim 22 wherein the analyte capture moiety is attached via one or more linker molecules.
25. A probe as claimed in claim 23 wherein the linker molecules comprises a poly amino acid or an alkane thiol.
26. A probe as claimed in claim 25 wherein poly amino acid is poly-L-lysine, poly-L-aspartic acid, poly-L-glutamic acid or mixtures of any other known aminoacids with the three aforementioned aminoacids.
27. A probe as claimed in claim 14 wherein at least one protein capturing moiety binds biotin or a bleomycin resistance protein.
28. A probe as claimed in claim 27 wherein the protein capturing moiety is streptavidin, avidin, neutravidin or bleomycin.
29. A probe as claimed in any of the preceding claims wherein the analyte capture moiety is provided in a layer which is otherwise substantially resistant to non specific protein binding.

30. A probe as claimed in claim 29 wherein the layer which is otherwise substantially resistant to non specific protein binding comprises a polymer or a self assembled monolayer (SAM) which is responsible for the generally protein repellant nature of the layer.
31. A probe as claimed in claim 30 wherein the polymer comprises polyethylene glycol (PEG), dextran, polyurethane or polyacrylamide.
32. A probe as claimed in claim 31 wherein the polymer is bound to the probe surface via one or more linker molecules.
33. A probe as claimed in claim 32 wherein the analyte capturing moiety is attached to the surface via the polymer and/or the linker molecules.
34. A probe as claimed in any of the preceding claims wherein a single common analyte capture moiety is provided on the surface.
35. A probe as claimed in any of claims 1 to 33 wherein a plurality of different analyte capturing moieties are provided on the surface.
36. A probe as claimed in any of the preceding claims further comprising a captured analyte.
37. A probe as claimed in claim 36 wherein the captured analyte comprises a protein.
38. A probe as claimed in claim 37 wherein the protein is a fusion protein.
39. A probe as claimed in claim 38 wherein the fusion protein comprises a biotin carboxyl carrier protein (BCCP).
40. A probe as claimed in claim 38 wherein the fusion protein comprises a phleomycin/zeocin resistance protein.

51. A method of producing a protein microarray for use with laser desorption ionization mass spectrometer comprising providing a probe as claimed in claim 14 and depositing protein in registration with the protein capturing moieties in the discrete target area.
52. A method of analysing a protein microarray as claimed in claim 51 subjecting the protein microarray to laser desorption/ ionisation mass spectrometry.
53. A method as claimed in claim 52 wherein the laser desorption/ ionisation mass spectrometry is matrix assisted laser desorption/ ionisation mass spectrometry MALDI.
54. A method as claimed in claim 53 wherein energy absorbing molecules are deposited over the whole surface or in registration with the discrete target area on which a protein has been captured.
55. A method as claimed in claim 54 wherein energy absorbing molecules are deposited in registration with the discrete target area on which a protein has been captured.
56. A method as claimed in claims 54 or 55 in which the energy absorbing molecules are deposited in a manner which denatures and thus unbinds the protein from the protein capturing moieties leaving the denatured protein in close proximity to the protein capture moiety on the surface.
57. A method as claimed in any of claims 54 or 55 wherein the energy absorbing molecules are present as a homogenous layer in the discrete target area in registration with the protein capturing moieties and captured protein.
58. A method as claimed in claim 57 wherein the homogenous layer is substantially continuous such that individual crystals are not visible at a 100 fold magnification and there are no visible gaps between neighboring crystals.

- a) providing a probe as claimed in claim 14;
- b) bringing said probe into contact with one or more proteins;
- c) performing laser desorption/ionisation mass spectrometry on the proteins on the surface of the probe.

68. The method of claim 67 which comprises between step b) and c) an additional step of removing unbound molecules from the probe by washing.

69. The method of claim 68 wherein said one or more proteins are contained in a mixture of proteins.

70. The method of any of claims 67 to 69 which is a method for identifying a protein on the surface of the probe and which comprises the additional steps of:

- d) determining the mass of the protein molecule;
- e) performing a digestion upon a replicate sample of said protein on a further probe or probe surface; and
- f) performing laser desorption/ionisation mass spectrometry on the peptides resulting from step e) to identify said proteins.

71. The method of any of claims 67 to 69 which is a method of analysing the function of a protein on the surface of the probe and a molecule interacting with said protein and which comprises the additional steps instead of step c) of:

- c) bringing a protein on the probe surface into contact with one or more test molecules;
- d) removing unbound test molecules from the probe surface;
- e) performing laser desorption/ionisation mass spectrometry on the protein and any bound molecule to determine the identity of the protein and/or test molecule.

72. The method of claim 71 wherein the test molecule is a small molecule, protein or nucleic acid.

73. The method of any of claims 67 to 69 which is a method of analysing the function of a protein and which comprises the additional steps of:

- c) bringing a protein on the probe surface into contact with one or more test substrates
- d) performing laser desorption/ionisation mass spectrometry on the protein and test substrates to determine the presence and/or identity of products of catalysis of said test substrates by the protein.

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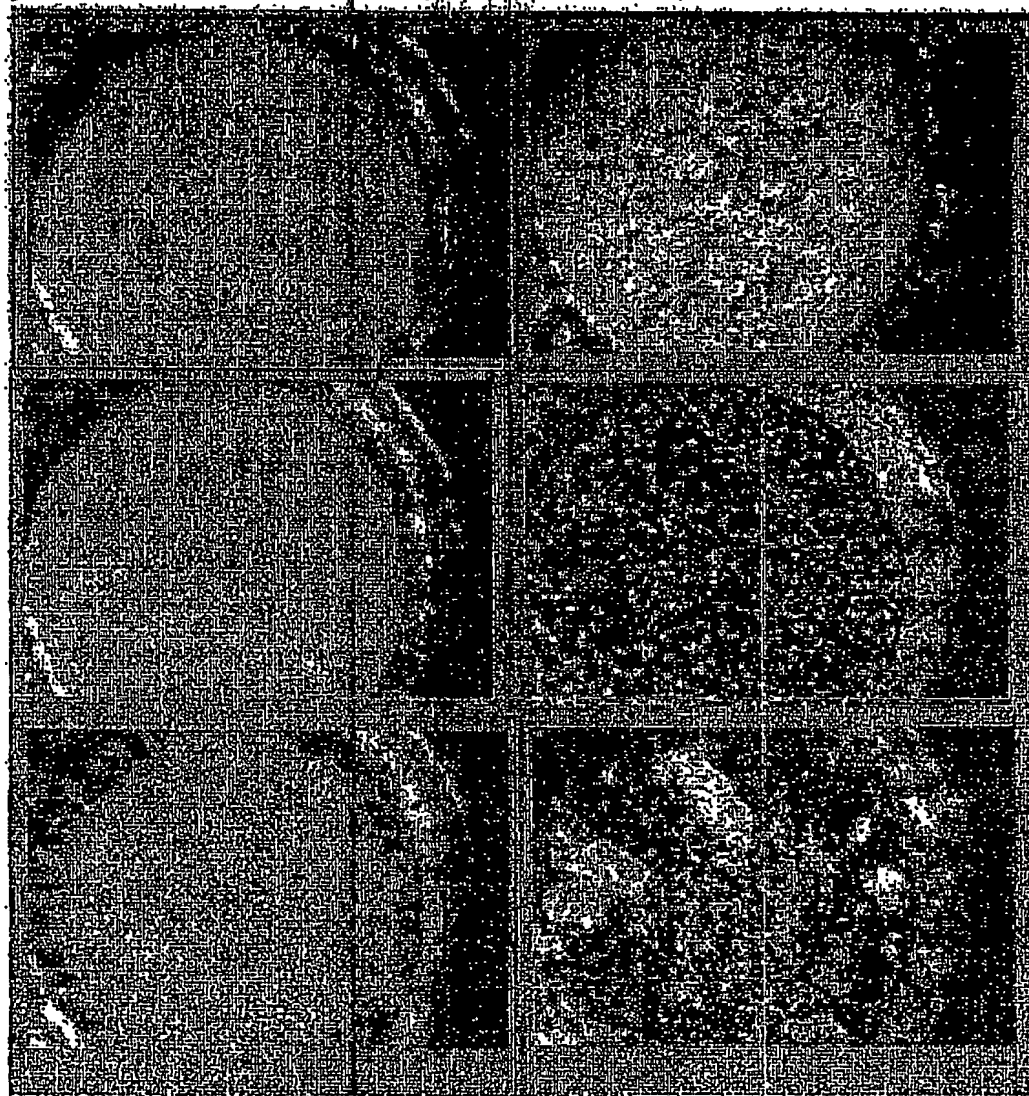
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Experimental Section

Figure 1) Matrix formulation for protein microarray.

a)



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b)

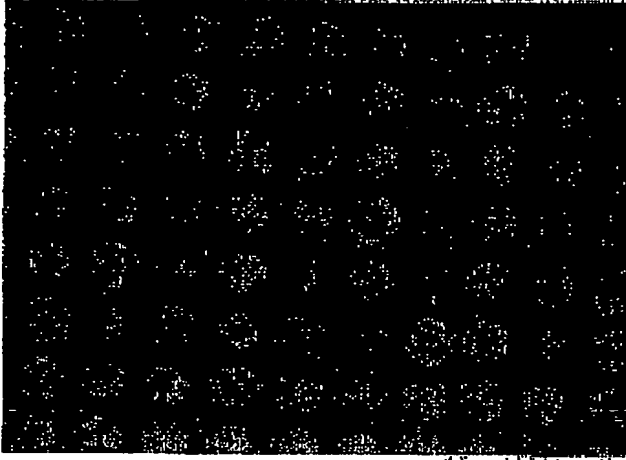
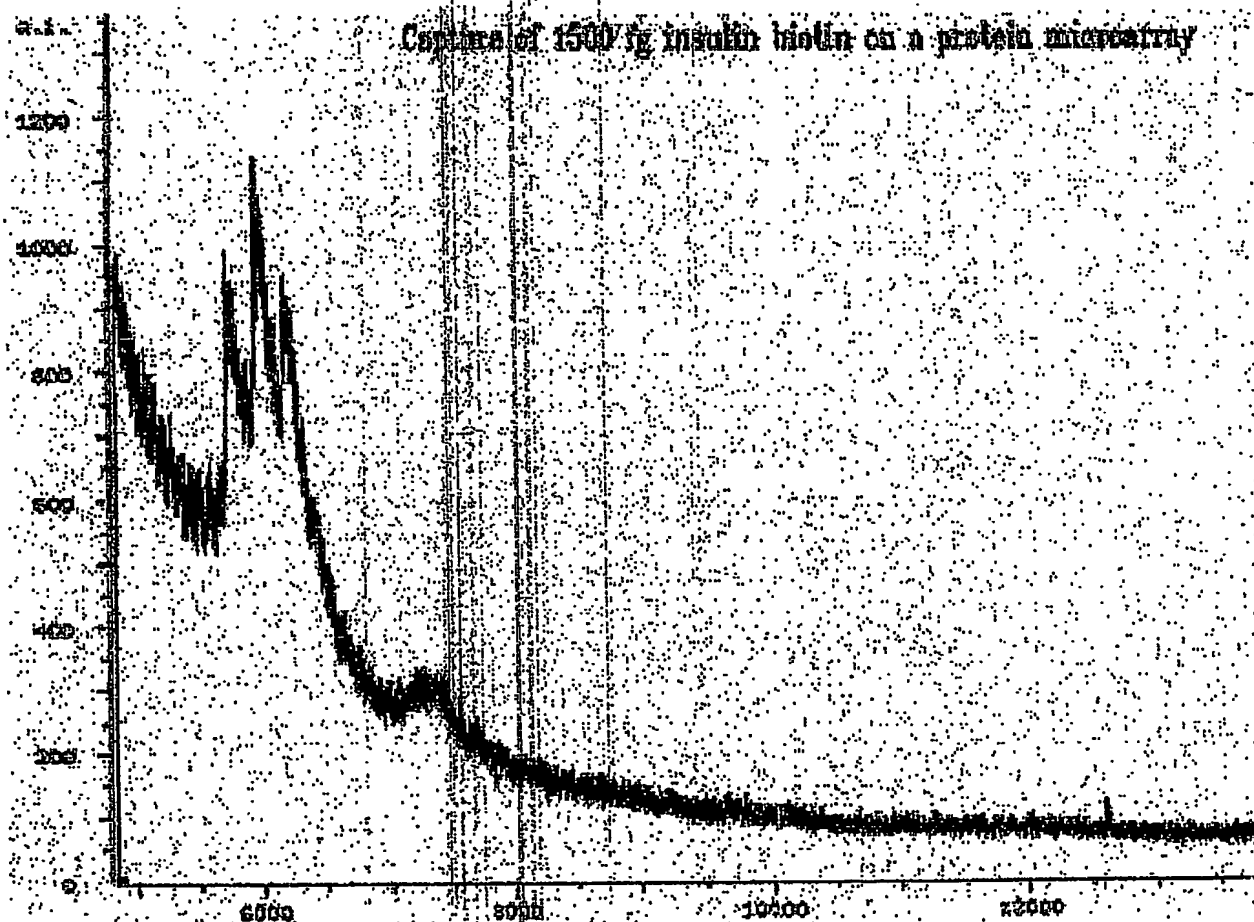


Figure 2) Insulin-biotin mass spectra acquired from a protein nano-array spotting down 1500 and 15 fg before washing.

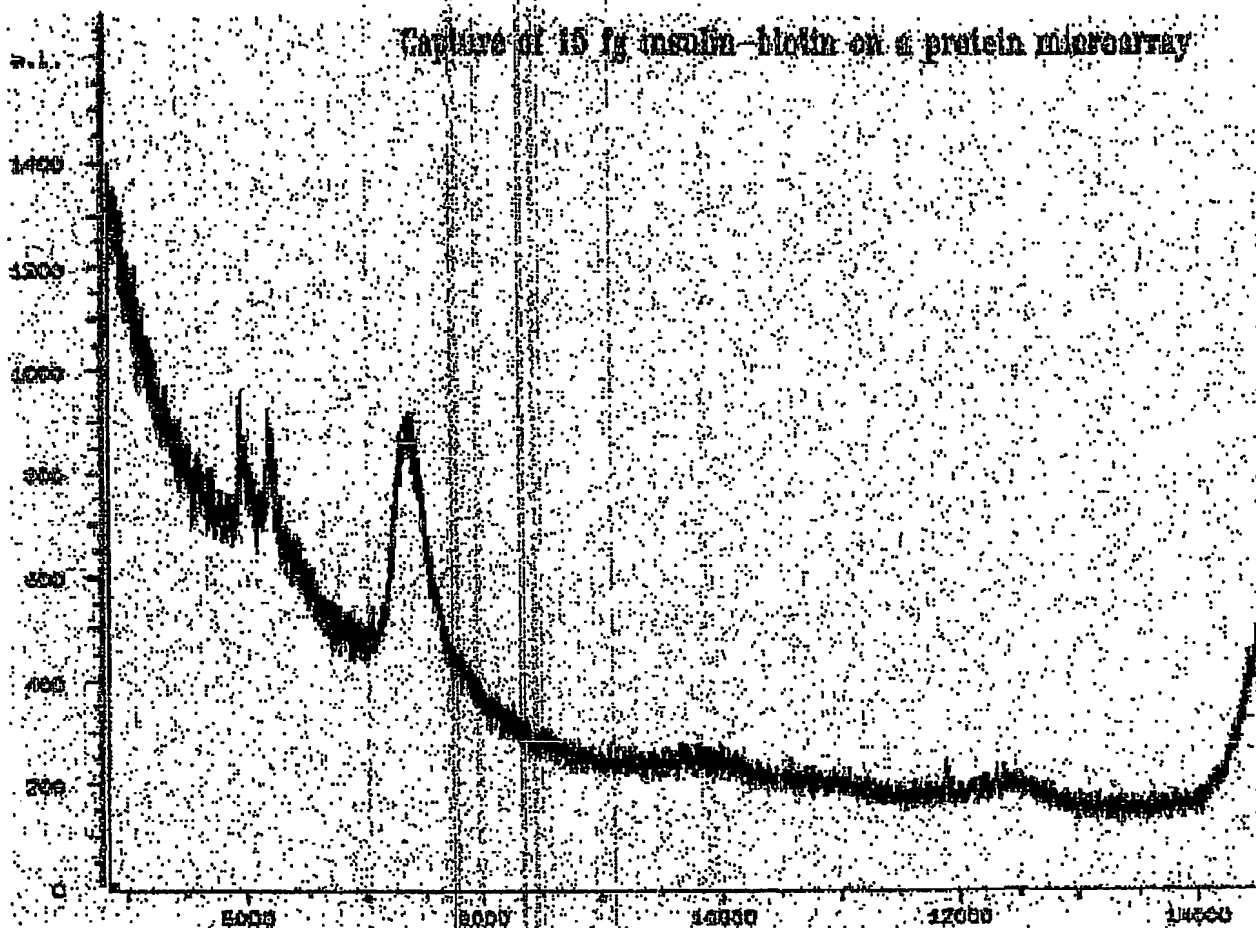
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a)



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b)

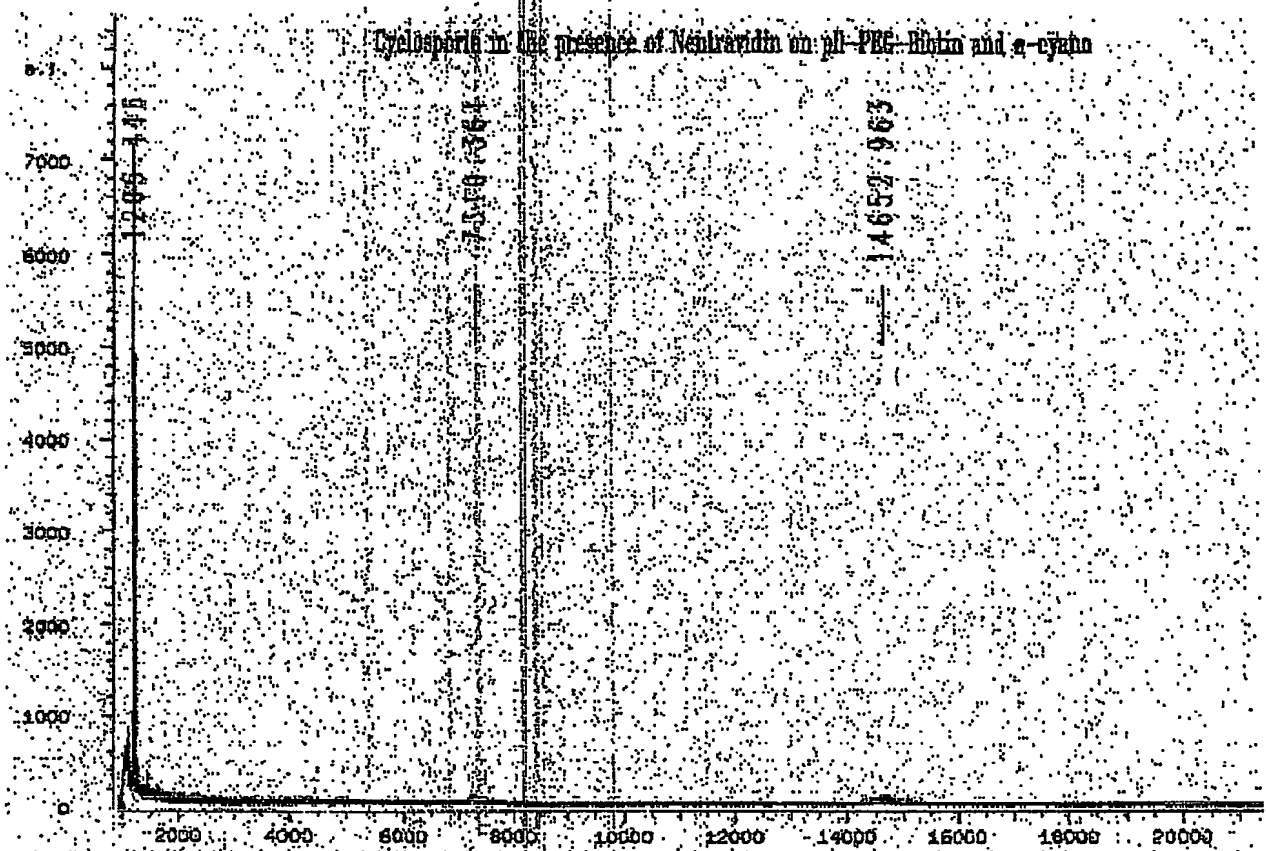


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Figure 3a) Cyclosporin detection in the presence of PEG-PLL-Biotin and neutravidin.



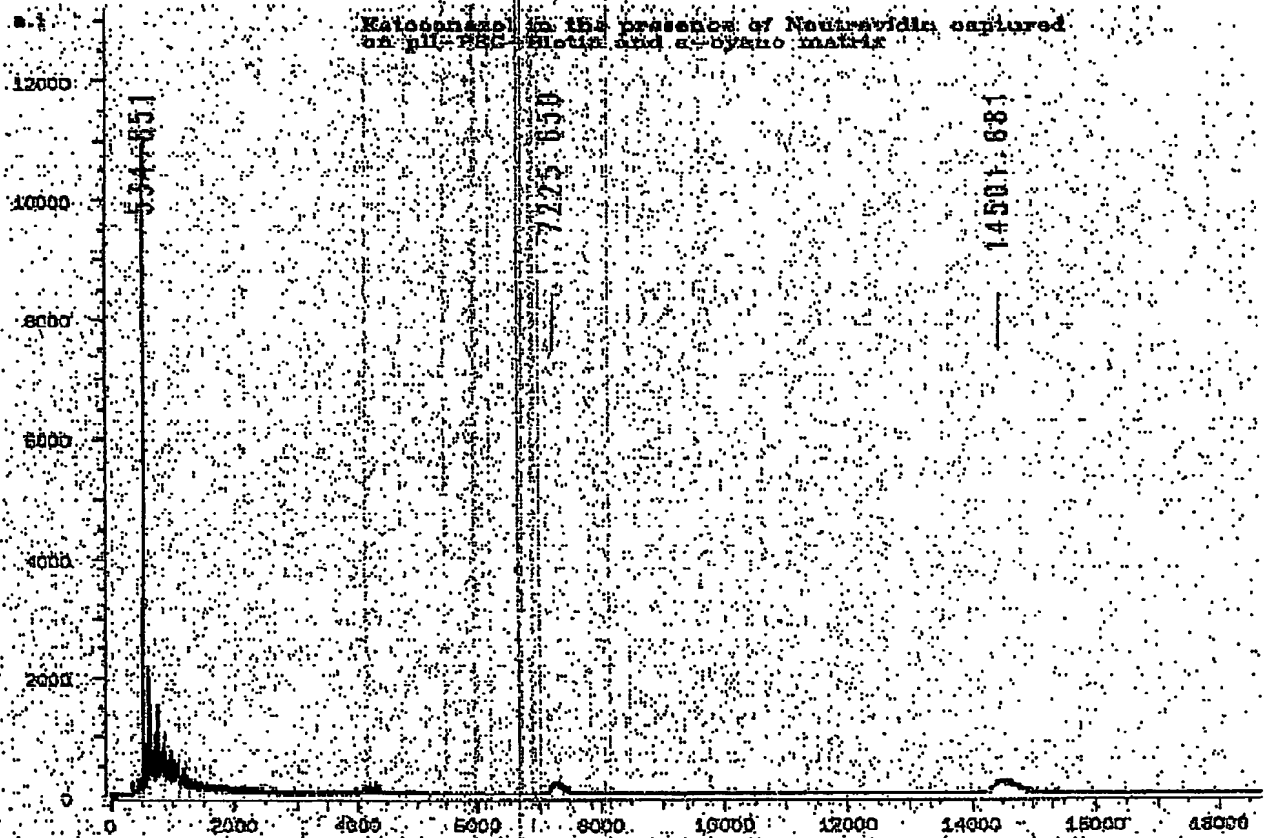
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Figure 3b) Ketoconazole detection in the presence of PEG-PLL-Biotin and neutravidin.

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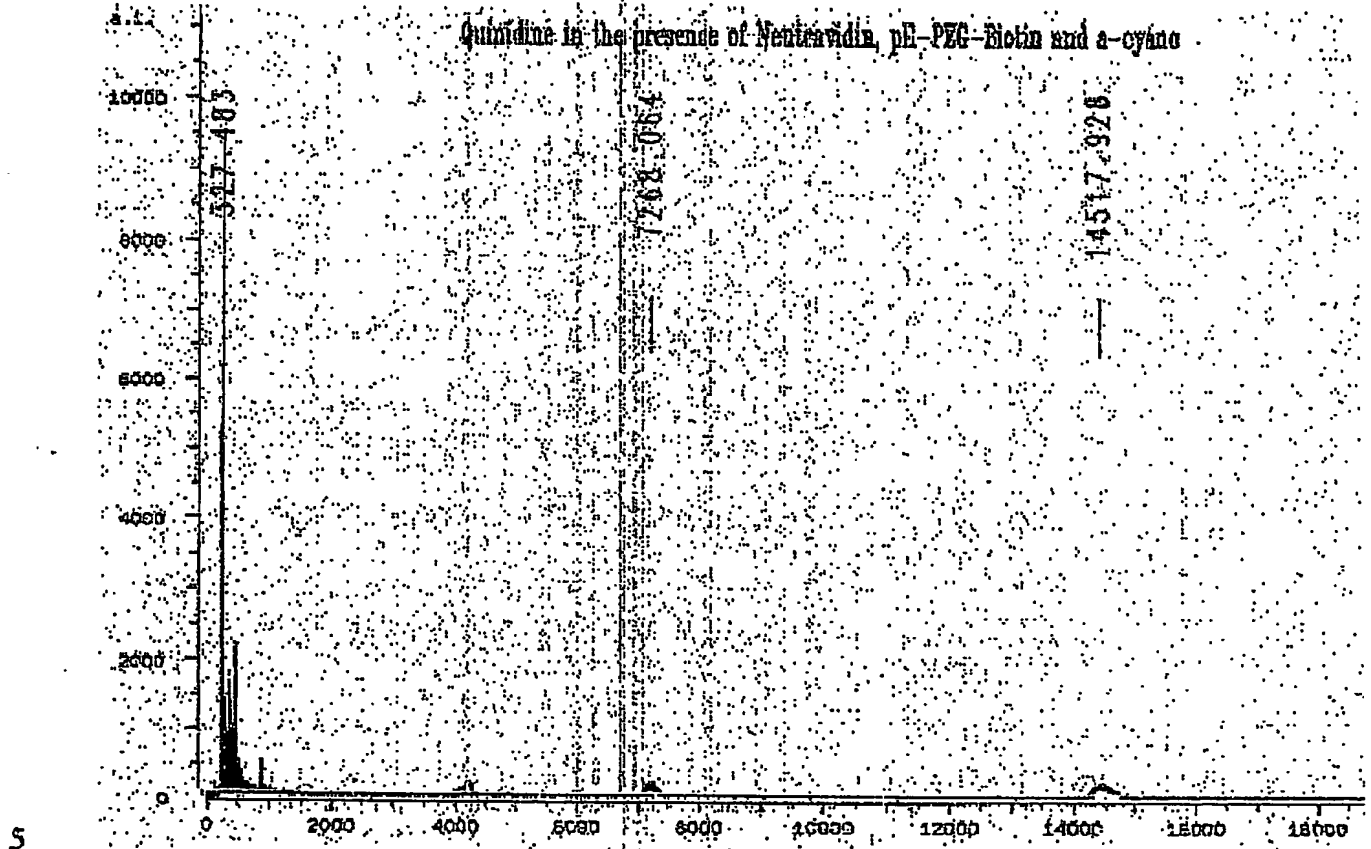


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Figure 3c) Quinidine detection in the presence of PEG-PLL-Biotin and neutravidin.



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Figure 4a) Detection of ADP and ATP with MALDI-TOF MS

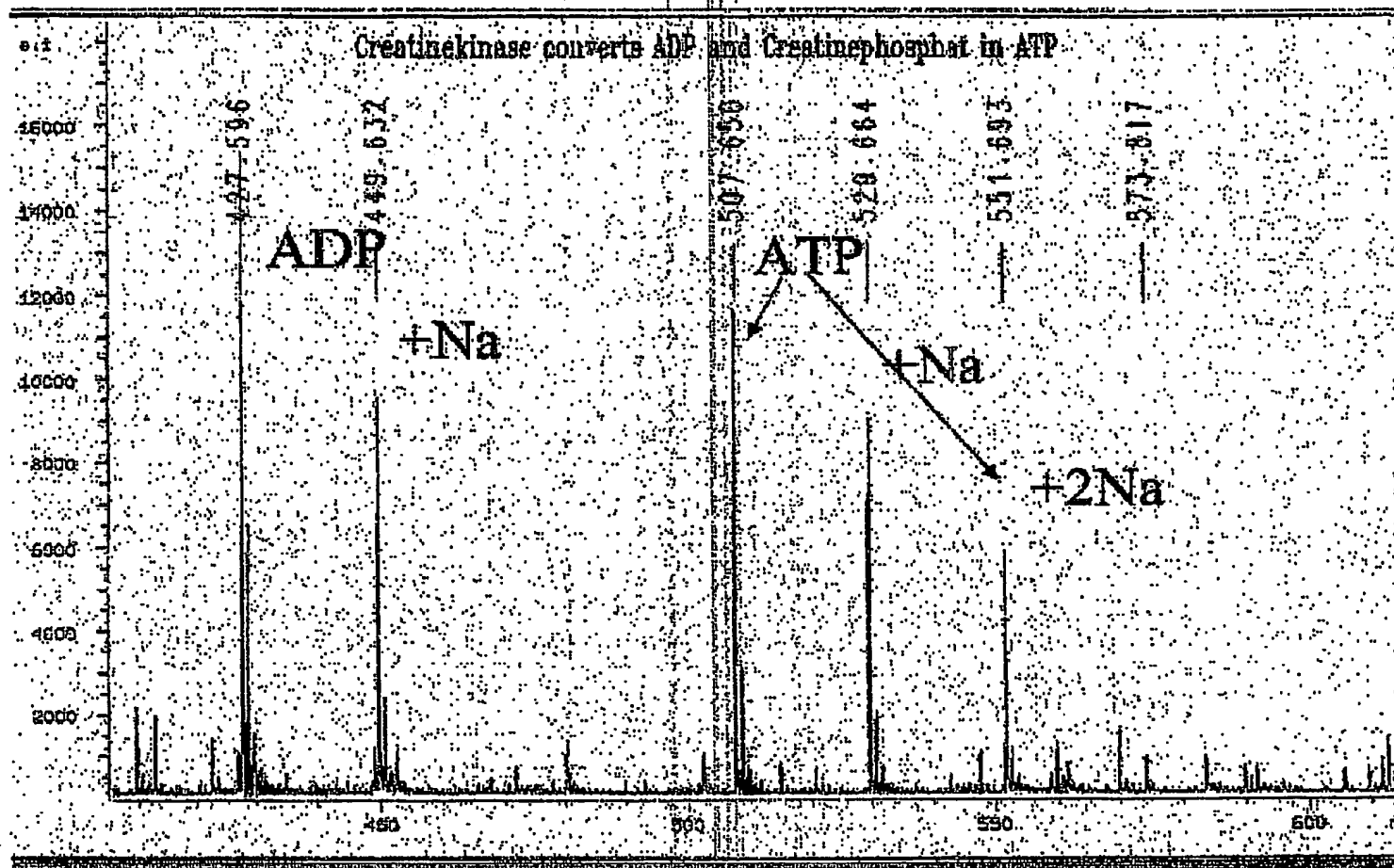
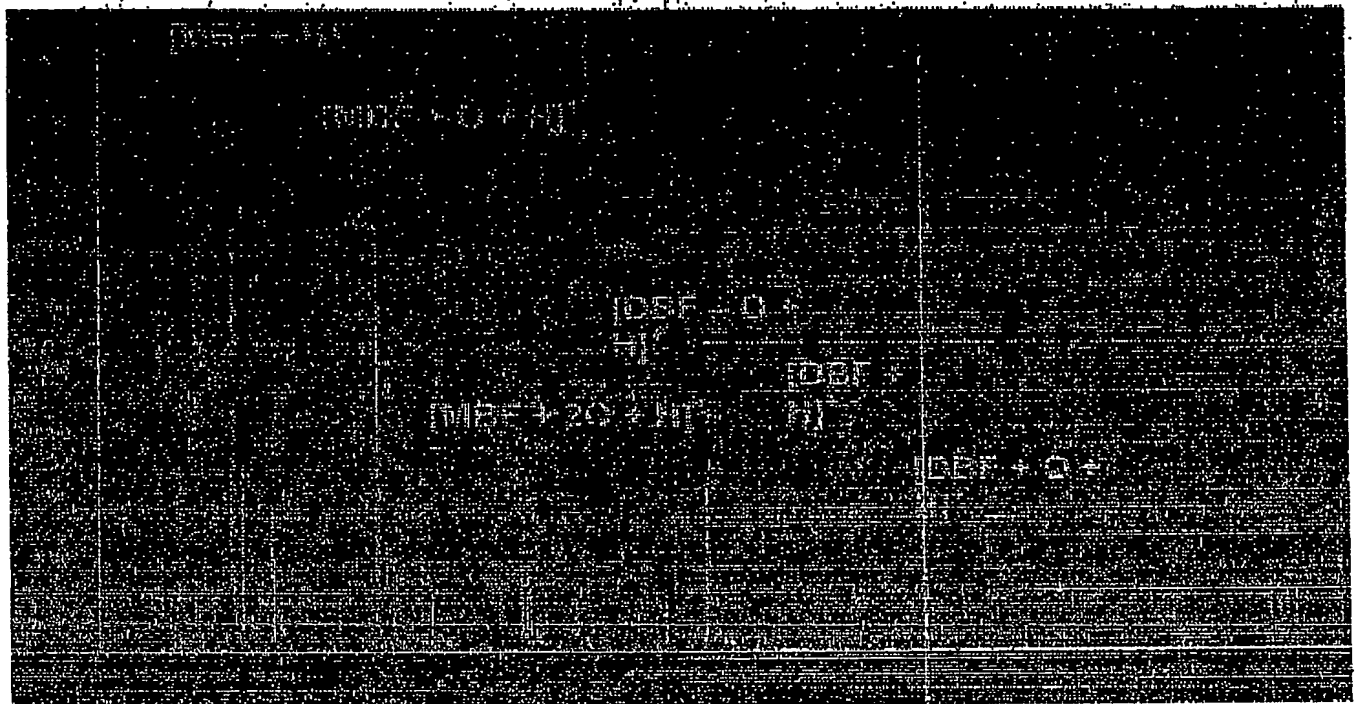
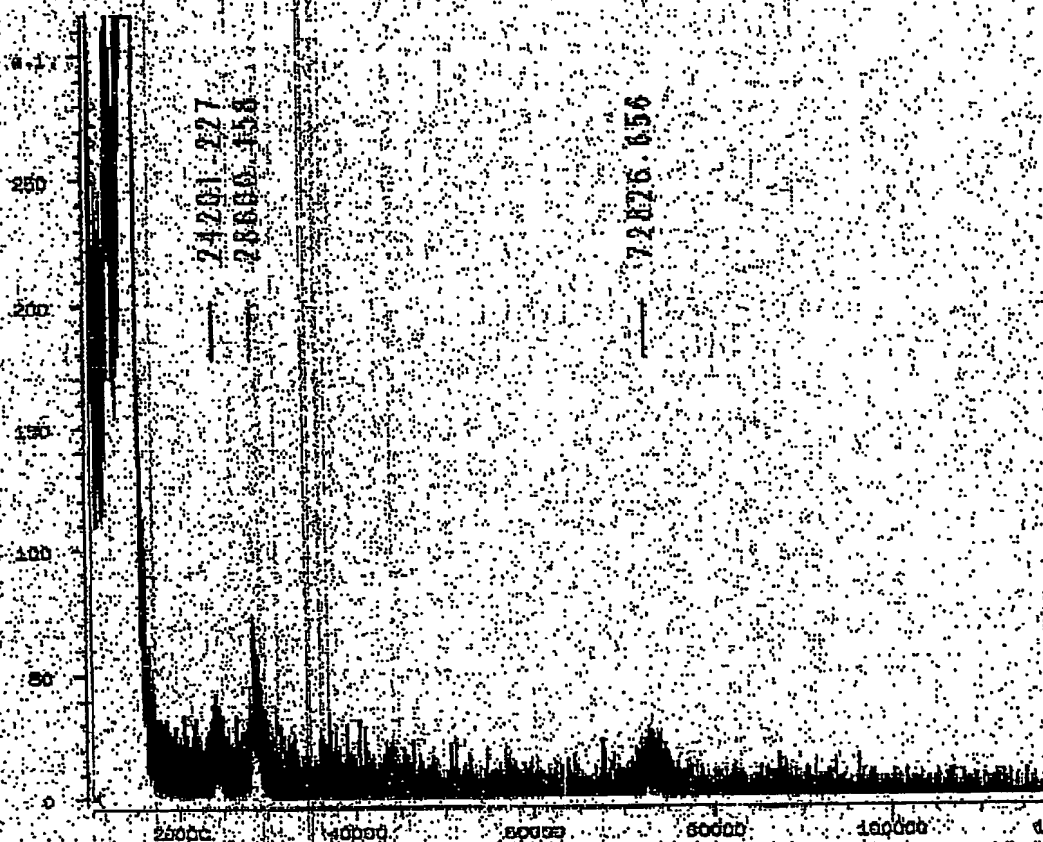


Figure 4b) Detection of human cytochrome P450-oxidation products



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Figure 5) Capture of 72 Kda biotinylated protein from a COVET experiment using a cardiac library



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Figure 6) Glutathione-S-Transferase-Biotin captured on Neutravidin coated MALDI glass target.

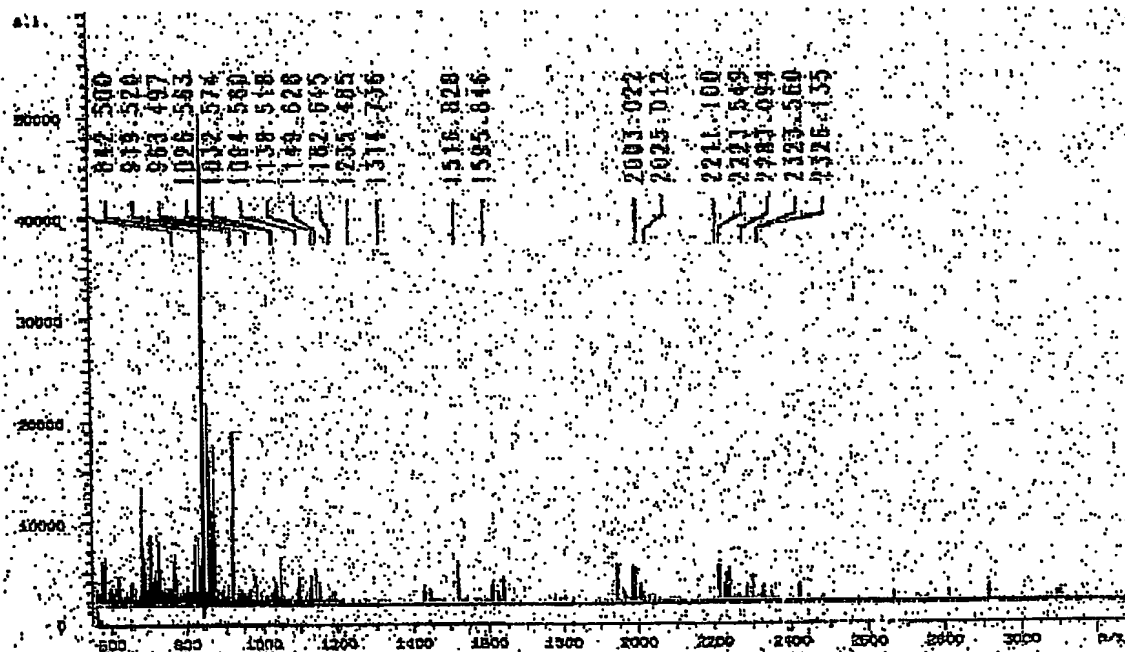
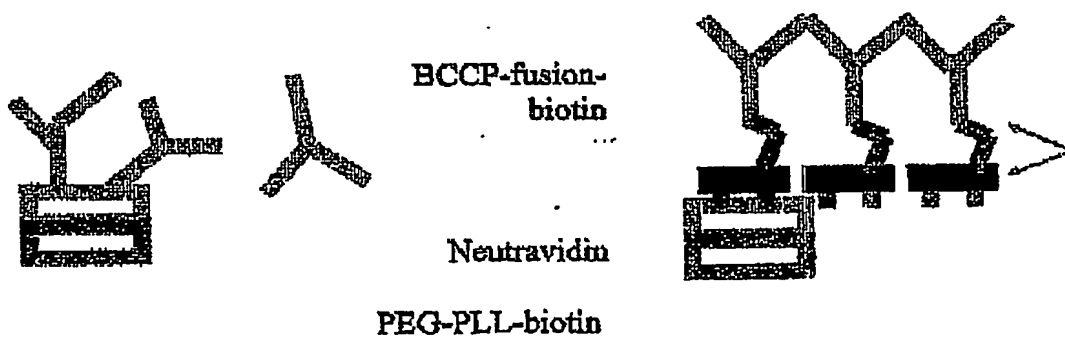


Figure 7) Coupling procedures for proteins on surfaces.

Random coupling

Ordered coupling

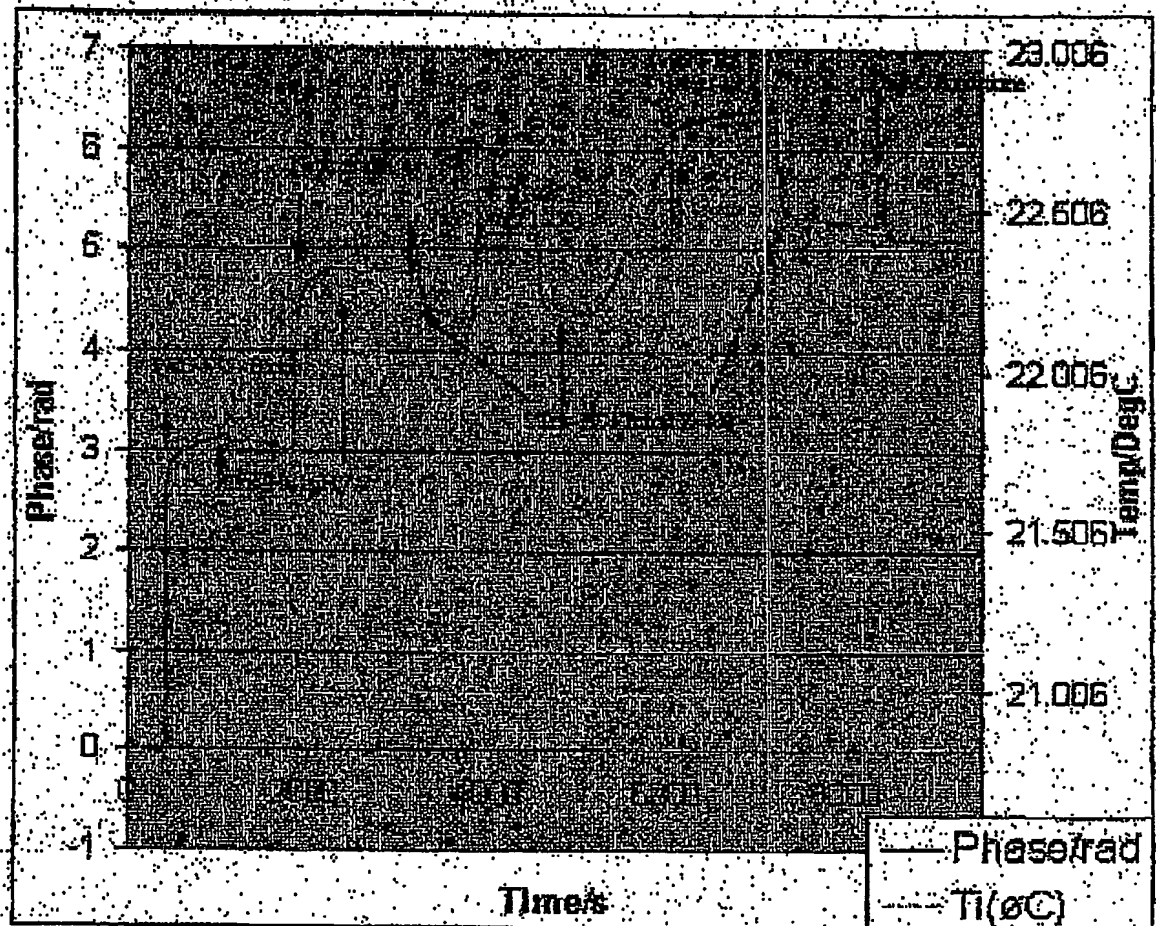


Amine coupling

PEG-PLL

Figure 8) Analysing PEG-PLL-Biotin affinity capture surface with a dual wavelength interferometer.

5



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Figure 9a

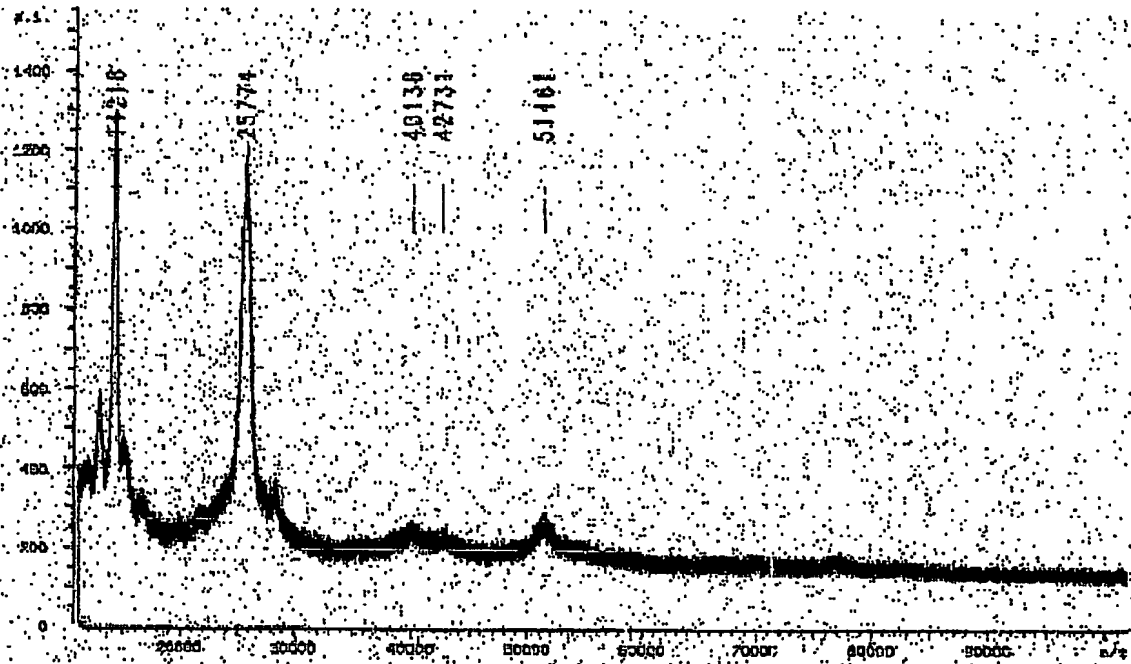
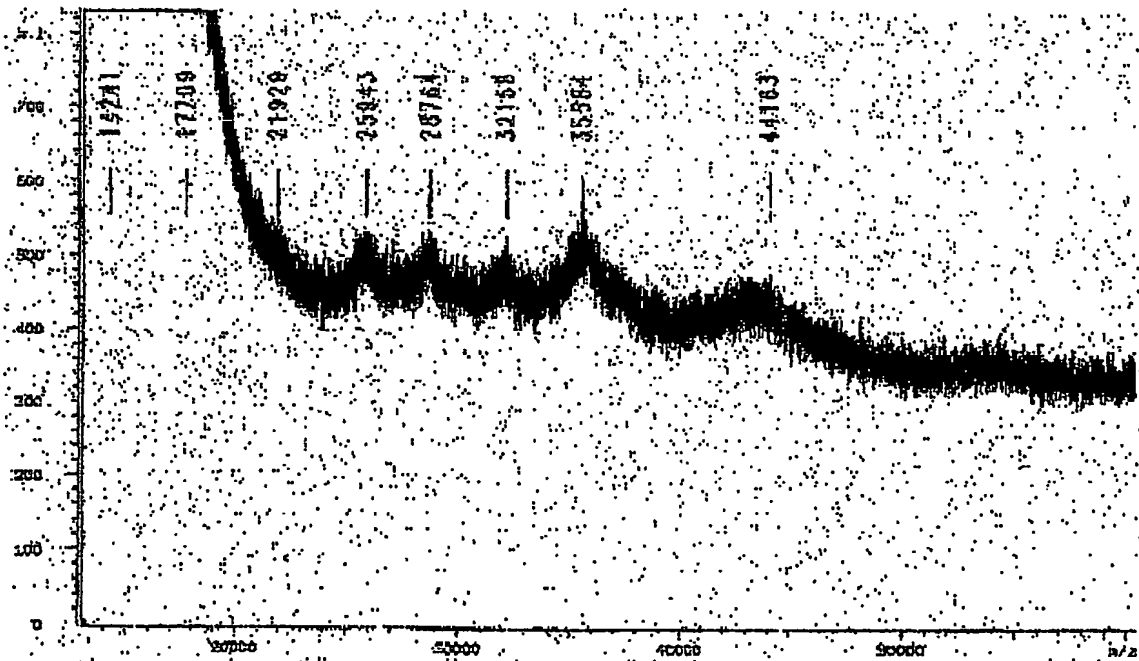


Figure 9b



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Figure 10a Mass spectrum of captured Lactose Rhodamine on PEG-PLL-biotin Neutravidin surface coated with Peanut lectin.

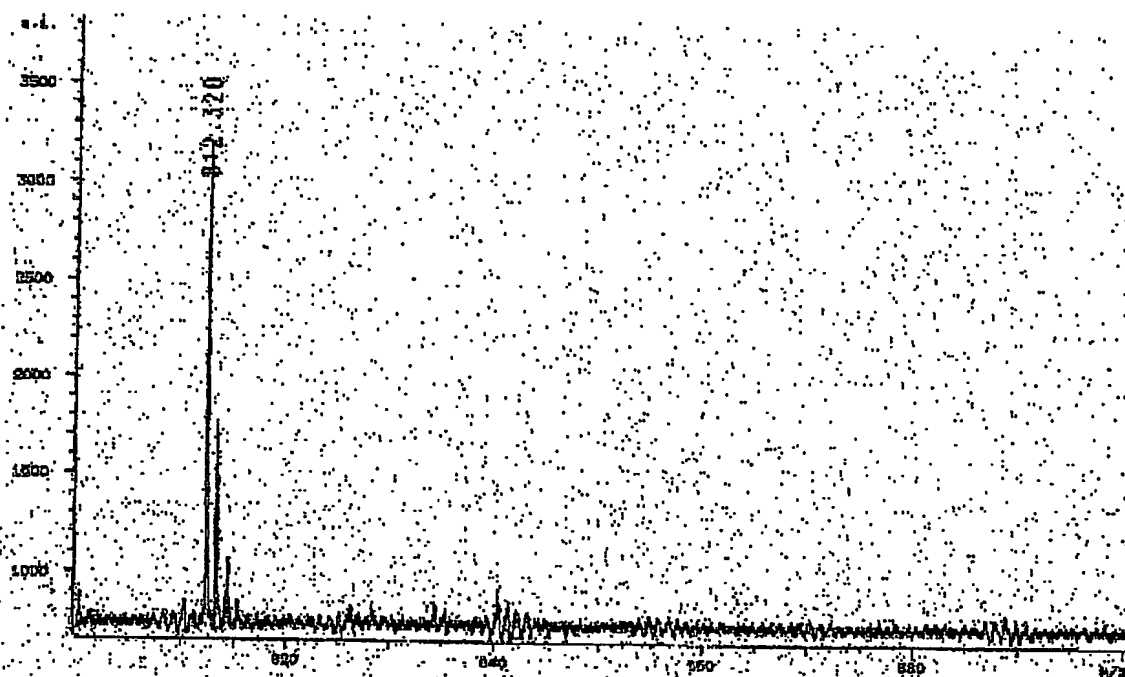


Figure 10 b Mass spectrum of Lactose Rhodamine prior capture on Peanut lectin

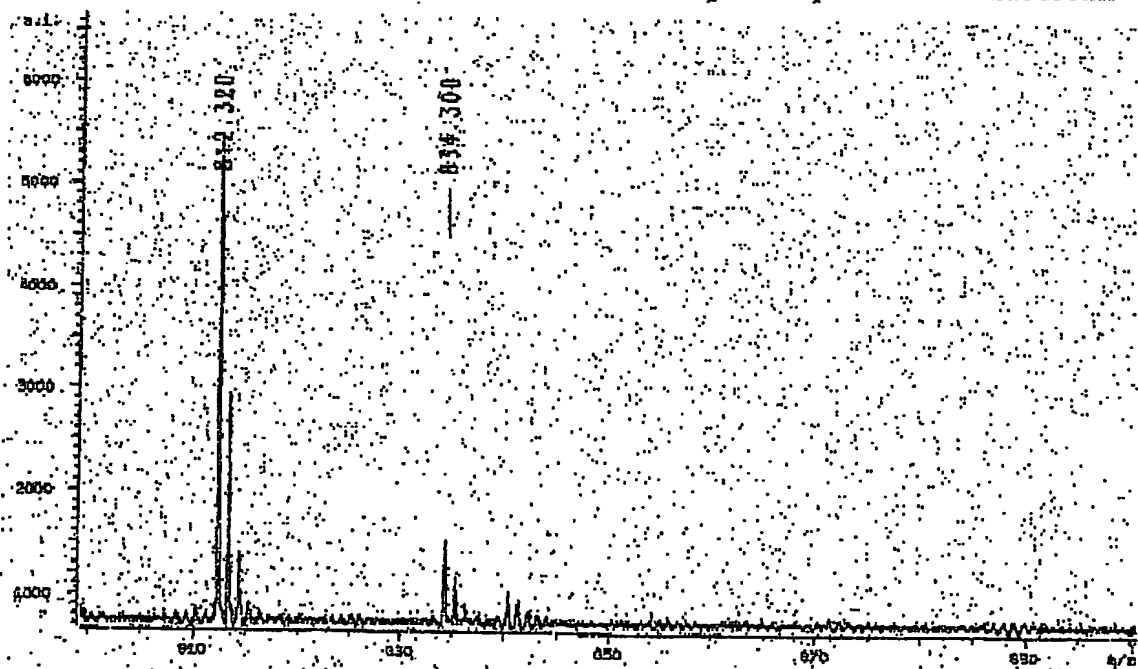
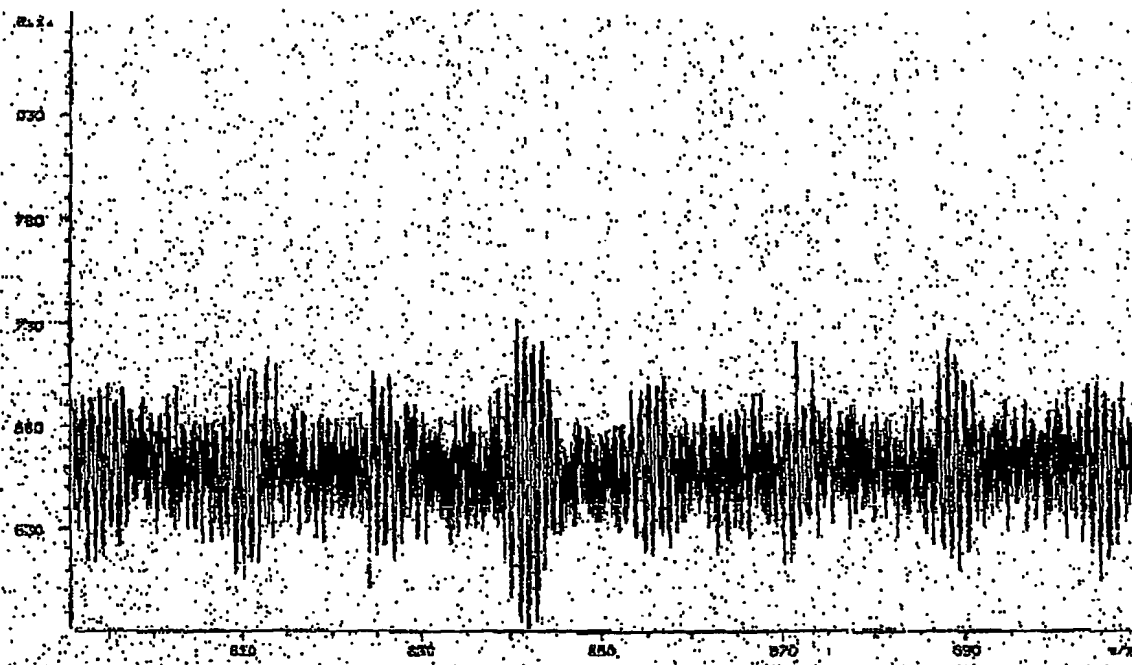


Figure 10c) Mass spectrum of FK506 binding protein that was immobilised onto a PEG-PLL-biotin neutravidin surface and probed with Lactose Rhodamin



5 Table 1

Peptide masses [M+H] ⁺	Glutathione-S- transferase	Trypsin	Avidin
770.416	X		
919.52	X		
963.497	X		
1026.563	X		
1032.574	X		
1094.56	X		
1138.518	X		
1149.628	X		
1182.645	X		
1314.736	X		
1516.828	X		
2326.1	X		
845.5		X	
2211.1		X	
2283.094		X	
919.53			X
1235.48			X
1595.845			X
2003.022			X

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